Function and targets of Fusarium oxysporum effectors
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The *Fusarium oxysporum* effector Six6 contributes to virulence and suppresses I-2 mediated cell death
Abstract

Plant pathogens secrete effectors to manipulate their host and facilitate colonization. *Fusarium oxysporum* f.sp. *lycopersici* (Fol) is the causal agent of Fusarium wilt disease in tomato. Upon infection Fol secretes numerous small proteins into the xylem sap (Six proteins). Most Six proteins are unique to *F. oxysporum*, but Six6 is an exception; a homolog is also present in two *Colletotrichum* species. *SIX6* expression was found to require living host cells and a knockout of *SIX6* in Fol compromised virulence, classifying it as a genuine effector. Heterologous expression of *SIX6* did not affect growth of *Agrobacterium tumefaciens* in *Nicotiana benthamiana* leaves or susceptibility of *Arabidopsis thaliana* towards *Verticillium dahliae*, *Pseudomonas syringae* or *F. oxysporum*, suggesting a specific function for Fol Six6 in the Fol-tomato pathosystem. Remarkably, Six6 was found to specifically suppress I-2 mediated cell death (I2CD) upon transient expression in *N. benthamiana*, while it did not compromise the activity of other cell death-inducing genes. Still, this I2CD suppressing activity of Six6 does not allow the fungus to overcome I-2 resistance in tomato, suggesting that I-2-mediated resistance is independent from cell death.

This chapter has been published as:
Introduction

Plants are challenged by a wide variety of microbes including viruses, bacteria, fungi and oomycetes (Agrios, 1997). Thanks to their multi-layered immune system plants recognise and defeat most potential invaders (Jones and Dangl, 2006). However, pathogenic microbes evolved various mechanisms that suppress plant immunity, resulting in disease (Win et al., 2012). Mostly, this disease-causing ability is mediated by secreted virulence factors - called effectors - that alter host-cell function or otherwise promote host colonization (Hogenhout et al., 2009). Therefore, identification and characterization of effectors provides insights into the mechanisms underlying susceptibility to pathogens.

Pathogenic strains of the fungus *Fusarium oxysporum* invade roots of a broad variety of plant species and cause wilt disease through colonization of the xylem vessels (Tjamos and Beckman, 1989). Each strain can only infect one or a few closely related hosts, and strains infecting the same host are grouped into the same host-specific form or *forma specialis* (f.sp.) (Armstrong and Armstrong, 1981; Katan, 1999; Katan and Di Primo, 1999). So far, most effectors have been identified from *F. oxysporum* strains infecting tomato and belonging to f.sp. *lycopersici*. The first *F. oxysporum* f.sp. *lycopersici* (hereafter called Fol) effectors that have been identified are proteins encoded by avirulence (*AVR*) genes, namely Avr1, Avr2 and Avr3 (Rep et al., 2004; Houterman et al., 2008; Houterman et al., 2009). Avr proteins trigger host resistance upon recognition by corresponding resistance (R) proteins in plants. Hence, Fol is divided into three races named after the corresponding R gene that recognises the Avr protein. Functional analyses revealed that Avr2 and Avr3 trigger resistance in tomato plants carrying respectively the R genes *I*-2 and *I*-3 (Rep et al., 2004; Houterman et al., 2009). Besides triggering resistance, both proteins are required for full pathogenicity on susceptible tomato lines (Rep et al., 2005; Houterman et al., 2009), classifying them as genuine effectors. Fol effector Avr1, recognised by I and *I*-1, does not contribute to virulence on susceptible cultivars, but suppresses *I*-2 and *I*-3 mediated resistance (Houterman et al., 2008). All three Avr proteins were originally identified in the xylem sap of Fol-infected tomato plants (Houterman et al., 2007). Besides the Avr proteins, eleven other secreted in xylem (Six) proteins have been identified so far; all are relatively small (<300 aa), generally cysteine-rich and produced with a signal peptide for secretion (Houterman et al., 2007; Lievens et al., 2009; Ma et al., 2010; Schmidt et al., 2013). Six proteins generally do not have sequence homology to proteins with known functions (Rep, 2005), prohibiting reliable prediction and assignment of specific functions to these proteins. Expression of at least some SIX genes (e.g. *AVR3, SIX2, AVR2* and *SIX5*) is regulated by the transcription factor Sge1; a knockout of the gene encoding this regulator impairs pathogenicity of the fungus and abolishes SIX gene expression (Michielse et al., 2009). Analysis of the
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Fol genome revealed that most SIX genes are localized on chromosome 14 (Ma et al., 2010; Schmidt et al., 2013). This lineage-specific chromosome is required for infection of tomato and can be shuttled via horizontal transfer from Fol to a non-pathogenic strain, converting this strain into a tomato pathogen (Ma et al., 2010). This highlights the importance of the SIX gene-carrying chromosome for the ability of the fungus to cause disease. Until now, Avr2 and Six5 have exclusively been found in Fol, suggestive of a tomato-specific function for these two effectors. For other Six proteins, such as Avr1, Six6, Six7, Six8 and Six9, close homologs are present in other formae speciales (Lievens et al., 2009; Chakrabarti et al., 2011; Thatcher et al., 2012), implying a more generic function for these proteins. Among these, Six6 is of special interest as homologs are also present in two other fungal plant pathogens: Colletotrichum orbiculare and C. higginsianum (Kleemann et al., 2012; Gan et al., 2013).

We here describe the functional characterization of Fol Six6, and show that it is a genuine effector protein important for pathogenicity.

Results

Fol Six6 homologs are present in six formae speciales of F. oxysporum and in two Colletotrichum species

Upon infection Fol secretes Six6 into the xylem sap of tomato (Lievens et al., 2009). The Six6 precursor is encoded by a gene spanning 648 bp (genbank accession ACY39286.1). SignalP 4.0 (Petersen et al., 2011) predicts a 16 amino acid (aa) signal peptide yielding a mature protein of 199 aa. Six6 contains seven cysteines and has a predicted molecular weight of 22 kD (ProtParam) (Wilkins et al., 1999). NCBI/BLAST searches did not reveal hits to proteins with known functions (data not shown), but uncovered the presence of Six6 in other F. oxysporum formae speciales. In total, six Six6 homologs were identified in respectively: F. oxysporum f.sp. melonis (Fom) (ACY39292.1), niveum (Fon) (ADO60274.1), radicis-cucumerinum (Forc) (ACY39291.1), passiflorae (Fop) (ADO60275.1), vasicum (For) (ADO60273.1) and cubense (Foc) (Lievens et al., 2009; Chakrabarti et al., 2011; Schmidt et al., 2013; our unpublished observations). The amino acid sequences of Fom, Fon and Forc Six6 are identical while the others diverge (Fig. 1). Fop Six6 shows the highest similarity with Fol Six6, having 93% sequence identity at the protein level. Six6 of Fom and Fov share, respectively, 90% and 89% of sequence conservation with Fol Six6. The most distantly related Six6 is that of Foc, having 60% identity. The number of non-conservative amino acid substitutions is overall very low – e.g. between Fol and Fom Six6, there is only one non-conserved change (Asn to Leu).

Outside the F. oxysporum species complex, Six6 homologs were found in C. higginsianum, called ChEC36, and in C. orbiculare. ChEC36 is 9 aa longer than the Six6 homologs from
Characterization of Six6

_F. oxysporum_ and has 38% protein sequence identity with Fol Six6. The Six6-like _C. orbiculare_ protein is 223 aa long and shares 57% identity with Fol Six6. Alignment of the six Six6 homologs (Fig. 1) shows that the N-terminal half of the mature protein is less conserved than the C-terminal half (10% vs. 33%). In the C-terminal half three conserved motifs (ExCVxRxLSxGK, GxxEGCTT, TxxYDxNxxPllxVxKlxyYxGEPG) can be discerned.

![Signal peptide](image)

**Figure 1:** Six6 homologs carry conserved motifs in their C-terminal part. Alignment of Fol Six6 and Six6 homologues from _F. oxysporum_ and two _Colletotrichum_ species. The signal peptide sequence predicted by Signal P is depicted, conserved amino acids are highlighted and conserved motifs are underlined. Fol-F._oxysporum_ f.sp. _lycopersici_, Fop-F._oxysporum_ f.sp. _passiflorae_, Fom-F._oxysporum_ f.sp. _melonis_, Fov-F._oxysporum_ f.sp. _vasinfectum_, Foc-F._oxysporum_ f.sp. _cubense_, ChEC36- _Colletotrichum higginsianum_, Co- _C. orbiculare_. * Since Six6 sequences of Fom, Fon and Forc are identical only the Fom sequence is shown.

In summary, Six6 is relatively widespread, and well conserved within the _F. oxysporum_ complex. Furthermore, homologs are present in _C. higginsianum_ and in _C. orbiculare_.

**SIX6 is expressed during early stages of Fol infection**

Six6 was originally identified in xylem sap of seven-week-old tomato plants, 2.5 weeks after infection with Fol (Houterman et al., 2007; Lievens et al., 2009). To determine whether _SIX6_ is also expressed during earlier stages of infection, RNA was collected from Fol-infected tomato seedlings 4, 8 and 12 days post inoculation (dpi). Expression
of \textit{SIX6} was monitored using RT-PCR and a \textit{SIX6} transcript could be detected at all selected time-points in infected plants, but not in mock-inoculated controls (Fig. 2A). Expression of \textit{SIX6} was not detected in Fol mycelium when grown in synthetic medium, but the transcript was found when grown in the presence of suspension-cultured tomato cells (Fig. 2B). In conclusion, \textit{SIX6} is expressed at early and late stages of host infection and its expression requires the presence of living host cells.

\textbf{\textit{SIX6} knockouts of Fol show reduced virulence}

To determine the role of Six6 in virulence, \textit{SIX6} knockout mutants were generated in Fol by replacing \textit{SIX6} with a hygromycin resistance cassette. PCR-based screening of 92 hygromycin resistant transformants identified 23 transformants that had undergone an \textit{in locus} recombination resulting in deletion of the \textit{SIX6} gene.

Six independent \textit{SIX6} knockout strains were selected for further analysis. To assess a possible contribution of \textit{SIX6} to virulence, 10-day-old susceptible tomato seedlings were inoculated with either: I) the \textit{SIX6} knockout strains, II) two independent ectopic transformants not having a \textit{SIX6} gene deletion, III) wildtype Fol (Fol007, race 2) or IV) water, to serve as a mock-control. Three weeks after inoculation plant weight and disease index were measured. The disease index (Rep et al., 2004) was scored on a scale from 0-4, with 0 having no symptoms (no wilting, no brown vessels) to 4 being either dead or small and wilted (Fig. S1 shows representative examples of the disease classes). Fig. 3A shows the disease index plotted against the plant weight of the Fol bioassay in a dot plot. The different treatments are clustered based on significant differences in diseases index (solid lines) or plant weight (dotted lines). Significant differences between the groups were determined in an ANOVA using a Fisher protected least significant difference (PLSD) at 95% resulting in three distinct classes for the disease index and five for weight (Tab. S1). As can be seen in Fig. 3A, all \textit{SIX6} knockout strains exhibited a significant increase in plant weight as compared to the wildtype Fol007 or the ectopic controls, indicating a reduction in fungal virulence. Surprisingly, however, a significant reduction in disease index was only observed for two of the six \textit{SIX6} knockout strains (\textit{ΔSIX6}-1 and \textit{ΔSIX6}-3 knockouts).
Figure 3:
Fol SIX6 knockout mutants are reduced in virulence. Susceptible tomato seedlings were inoculated with wildtype Fol (Fol007), strains in which SIX6 was deleted (ΔSIX6-1-6), or a knockout strain (ΔSIX6-3) in which SIX6 was re-introduced (ΔSIX6-3+SIX6-1-5). As a control mock-inoculated plants were used (mock) and two transformants (ect), in which the SIX6 deletion construct was integrated ectopically. (A) Average plant weight of 20 plants is plotted against the average disease index (Fig. S1) of the same plants. Deletion of SIX6 impairs pathogenicity as shown by the increased plant weight as compared to infection with wildtype or the two ectopic transformants. (B) Two of the five independent knockout strains carrying the SIX6 complementation construct display a similar average disease index and plant weight as the Fol wildtype. Error bars represent the standard error. Clustering is based on an ANOVA (Fisher PLSD significant at 95%) (Tab. S1) using either the disease index (solid line) or plant weight (dashed line).

To ascertain that the reduced virulence of the mutants was indeed caused by loss of SIX6, mutant ΔSIX-3 was transformed with a SIX6 complementation construct. PCR confirmed the presence of an ectopically inserted construct in five transformants and virulence of these strains was assessed using bioassays. One transformant (ΔSIX6-3+SIX6-4) showed less disease symptoms than the SIX6-3 knockout. This impairment
in virulence was possibly due to a mutation caused by the ectopic insertion of the SIX6 construct or an unlinked mutation due to the transformation procedure. Statistical analysis (Anova with PLSD at 95%) showed that two transformants (ΔSIX6-3+SIX6-1 and ΔSIX6-3+SIX6-3) cluster together with the SIX6 knockout with regard to the disease index and the plant weight, indicating no complementation of virulence (Fig. 3B). However, in the two other SIX6 transformants (ΔSIX6-3+SIX6-2 and ΔSIX6-3+SIX6-5) pathogenicity was fully restored. Fig 3B, shows that these two complementants cluster with the wildtype Fol007 and have both a high disease index and a strong reduction in plant weight, confirming full restoration of virulence (Fig. 3B). Hence, the reduction in virulence of the SIX6 knockout can be functionally complemented using an ectopically expressed SIX6 gene.

To analyse the expression of SIX6 in the SIX6-3 knockout and the derived complementants, RT-PCR was performed on RNA isolated from roots of infected tomato seedlings (Fig. 4). To assess the presence of fungal RNA, confirming successful Fol infection, FEM1 was used as a positive control. A FEM1 transcript was detected in cDNA from all inoculations except for the mock control. Notably, the intensity of the FEM1 bands varied for the respective complementation lines and in general the strains exhibiting the strongest increase in plant weight (ΔSIX6-3 and ΔSIX6-3+SIX6-4) showed the lowest amount of FEM1 amplification product, indicative for a lower abundance of the fungus in these infected plants. Next, expression of SIX6 was analysed, and as shown in Fig. 4, a SIX6 amplification product was found for wildtype Fol007 and all SIX6 complementants, but not for the ΔSIX6-3 strain, further confirming that it is a genuine knockout. Since the control without reverse transcriptase (-RTase) did not show a SIX6 amplification product it can be excluded that the bands originate from contaminating genomic DNA. In conclusion, since deletion of SIX6 impairs Fol pathogenicity, which can be restored by ectopic complementation, SIX6 can be considered a genuine Fol effector gene.
Six6 does not enhance virulence of Agrobacterium tumefaciens in Nicotiana benthamiana, nor alters susceptibility to Verticillium dahliae or F. oxysporum in Arabidopsis thaliana

To test whether the virulence-promoting activity of Six6 extends to pathogens other than Fol, its ability to enhance virulence of a bacterial pathogen was assessed. The disarmed *A. tumefaciens* strain GV3101 was transformed either with a binary vector encoding solely a TAPi-tag (tandem affinity purification-tag), or with the same vector carrying a C-terminal TAPi-tagged SIX6. The sequence encoding the endogenous signal peptide of Six6 was removed (dSPSIX6) to enforce a cytosolic localisation of the protein. As a positive control the *Pseudomonas syringae* effector gene AvrPto was used. When *AvrPto* is expressed in *N. benthamiana* leaves using *A. tumefaciens* its bacterial growth was increased (Hann and Rathjen, 2007).

When grown in liquid culture no growth differences were found between *A. tumefaciens* carrying the empty TAPi binary vector or vectors containing SIX6 or AvrPto (data not shown), showing that the mere presence of these transgenes did not affect bacterial growth. *In planta* growth of the transformed bacteria was monitored at two time-points after infiltration in *N. benthamiana* leaves (Fig. 5A). *A. tumefaciens* containing the empty TAPi vector typically grew 1 log cfu cm\(^{-2}\) in 4 days. Expression of SIX6 did not alter growth of *A. tumefaciens in planta*; the SIX6 transformants grew at the same rate and to the same titer as the empty vector control (1 log cfu cm\(^{-2}\)/2 days) (Fig. 5A). The positive control, *A. tumefaciens* carrying *AvrPto*, grew both faster and to higher titers, >2 log cfu cm\(^{-2}\)/2 days. The lack of a Six6 induced phenotype is not due to the lack of SIX6 expression as the protein could readily be detected on a Western blot probed with a PAP antibody recognising the TAPi-tag fused to Six6 (Fig. 6B). Hence, Six6 does not promote growth of *A. tumefaciens* in *N. benthamiana*.

To test whether Six6 alters susceptibility to other plant pathogens, stably transformed *Arabidopsis thaliana* Col-0 plants were generated expressing SIX6. Expression of the SIX6 gene was driven by the 35S promoter and to aid detection of the Six6 protein it was fused to a TAPi-tag. Two independent transgenic lines (Six6-1 and Six6-2) containing a single insertion of the 35S::dSPSIX6-TAPi construct were selected for analysis. The SIX6 over-expressing lines did not exhibit morphological aberrations nor showed a phenotype distinct from non-transformed Arabidopsis when grown under standard conditions (Fig. 5B, compare top panels). Accumulation of the SIX6 protein in the two transformants was confirmed by Western blotting (Fig. 5C). Six6 is present in many *F. oxysporum* strains (Fig. 1), but not in the Arabidopsis-infecting strain Fo5176 (Thatcher et al., 2012). Hence, this strain was used to assess a potential change in disease susceptibility to *F. oxysporum* in Arabidopsis due to expression of SIX6. Thirteen-day-old seedlings were infected with Fo5176 and disease index was scored on a scale from 0
to 5, in which 0 means that no disease symptoms developed and 5 that plants are dead (Tab. S2), at either nine or 15 days after infection (Fig. 5D). No significant difference (p > 0.05) between disease symptoms on wildtype Col-0 and Col-0 35S::dSP/SIX6-TAPi was observed, leading us to conclude that Six6 does not alter susceptibility of the plant to this strain of *F. oxysporum*.

**Figure 5**: SIX6 does not alter growth of *A. tumefaciens* in *N. benthamiana* or susceptibility to *V. dahliae, F. oxysporum* or *P. syringae*. (A) *In planta* growth of *A. tumefaciens* carrying respectively SIX6, a positive control (*AvrPto*) or an empty vector (TAPI). Samples were taken 0, 2 or 4 days after *N. benthamiana* leaf infiltration.
Bacterial growth of the empty vector control and SIX6 did not differ, while the bacterial titer was significantly higher in the AvrPto control. Bars indicate standard deviation of three replicates. (*) indicates significant difference between TAPI and AvrPto in a student t-test (2 dpi p=0.0006, 4dpi p=0.02). (B, D and E) Bioassays were performed on Col-0 and two independent Col-0 35S::dSPSIX6-TAPI (Six6-1 and Six6-2) transformants. (B) Photographs showing disease development (chlorosis and slightly reduced stature) of three representative plants 23 days after mock (upper rows) or V. dahliae inoculation (lower rows). (C) Western blot showing the presence of the TAPi-tagged Six6 protein in A. thaliana leaf protein extracts of the Six6 transformants. Detection was done using the PAP antibody. M: protein marker (D) Disease index (Tab. S2) scored at 9 and 15 days after F. oxysporum infection. Bars indicate standard deviation of 24 replicates. (E) Bacterial growth of P. syringae three days after infiltration (dpi). Bars indicate standard deviation of eight replicates. (*) indicates significant difference between the wildtype Col-0 and the Six6-2 transformant in a student t-test (p=0.02).

To assess a potential contribution of Six6 to susceptibility of Arabidopsis towards another wilt-causing fungus, bioassays were performed using Verticillium dahliae. 21-day-old wildtype plants and the two SIX6 expressing A. thaliana lines were inoculated with V. dahliae spores and disease symptoms where scored 23 days after inoculation (Fig. 5B). The amount of chlorosis was scored (Materials and Methods) as a measure to quantify disease symptoms. As can be seen in Fig. 5B, disease symptoms were relatively weak and Six6 did not enhance or reduce symptom development on A. thaliana.

Finally, in planta growth of the plant-pathogenic bacterium P. syringae DC3000 was analysed. 22-day-old A. thaliana plants were syringe-infiltrated with P. syringae and leaf discs were collected from the infiltrated area at 3 dpi. A small, but significant, decrease in bacterial growth was observed (< 1 log cfu cm$^{-2}$) in the 35S::dSPSIX6-TAPI line Six6-2 compared to the untransformed line, but no difference was observed in line Six6-1 (Fig. 5E).

In summary, transient SIX6 expression does not increase susceptibility of N. benthamiana to A. tumefaciens, nor does SIX6 expression in A. thaliana enhance susceptibility to Fo5176 or V. dahliae. Furthermore, susceptibility of A. thaliana towards P. syringae was not consistently affected by expression of SIX6.

Six6 suppresses I-2 mediated cell death

Many effectors enhance virulence of a pathogen by suppressing cell death, a response that often accompanies plant defence (Guo et al., 2009; Stassen and Van den Ackerveken, 2011). To test whether Six6 can interfere with cell death, the SIX6 gene was co-expressed with a set of cell death-inducing genes using agro-infiltration in N. benthamiana leaves. Since many R proteins can trigger cell death, representative members from different R gene families were selected. Four distinct solanaceae R genes (Rx, Pto, Cf4 and I-2) that respectively confer resistance to a viral, a bacterial and two fungal pathogens, were selected for analysis (Table 1). Co-expression of these R genes with their corresponding AVR genes triggers a cell death reaction generally referred to as a hypersensitive response (HR). As a positive control for a cell death suppression assay we tested whether Fol
Avr1 can be used. We reported before that Fol Avr1 suppresses I-2 mediated resistance of tomato (Houterman et al., 2008). To test whether Avr1 can suppress I-2 mediated cell death (I2CD) the I-2 gene was co-expressed with an AVR1 construct that lacks its signal peptide and prodomain (dPDAVR1) to assure that both proteins co-localise in the cytosol. In Fig. 6A, the suppressing effect of Avr1 on I2CD is compared with the effect of Six8, a Fol Six protein with a size similar to Avr1 and Six6. In contrast to Six8, Avr1 was found to suppress cell death and reduce necrosis of the overlapping area. To better visualise the contrast between the living green cells and the dead brown cells the dead cells were stained with Trypan Blue. As can be seen in Fig. 6A (right panel), the region where AVR1- and I-2/AVR2-expression overlaps showed a slightly reduced blue staining as compared to the Six8 control (Fig 6A). This is not due to a lack of expression as Western blot analysis shows accumulation of Six8 and the other two effectors (Fig 6B). So, besides its suppressive effect on I-2 mediated resistance, Avr1 also suppresses I2CD, which makes it a proper positive control for a cell death suppression assay.

**Figure 6**: Six6 suppresses I2-mediated cell death and ion leakage. Co-expression of AVR1, SIX6 and SIX8 with AVR2 and I-2. (A) Pictures of N. benthamiana leaves taken 48 hours after infiltration (hpi) (left panel) and visualization of cell death with Trypan Blue staining (right panel). Lower circle: agro-infiltration of I-2 and AVR2, upper circle: agro-infiltration of AVR1, SIX6 and SIX8 respectively. Effect on I-2 mediated cell death was scored in the overlap of the circles. (B) Accumulation of Avr1, Six6 and Six8 shown using a Western blot probed with the PAP antibody (all Six proteins carry a TAPI tag). Arrows indicate the expected position of the Avr1-, Six6- and Six8-TAPI fusion proteins. Ponceau S staining shows equal loading. M: protein marker. (C) and (D) conductivity of N. benthamiana leaf discs sampled from infiltrated leaf areas co-expressing I-2, AVR2 and
Characterization of Six6

AVR1 (C) or I-2, AVR2 and SIX6 (D). Leaf discs were collected at 26 hpi and conductivity was monitored at 0, 2 and 8 h after harvesting and compared to that of I-2, AVR2 and AVR2\textsuperscript{I-2-} expressing regions present on the same leaf. Bars represent standard deviation of five replicates. (*) significant difference between the AVR2\textsuperscript{I-2-} control and AVR1 (p=0.04) or SIX6 (p=0.002) in a student t-test.

Notably, SIX6-infiltration reduced I2CD to an even larger extent than Avr1, as exemplified by the reduced Trypan Blue staining (Fig 6A, lower panel). Since the SIX6 construct does not carry the sequence encoding the signal peptide (dSP\textsubscript{SIX6}) this implies that the Six6-mediated suppression of the I2CD occurs inside the plant cell. Its cell death suppressive effect was exclusively observed for I-2 as Rx-, Pto- and Cf4-mediated HR were unaffected by Six6 (Table 1). Next, we analysed whether Six6 can also suppress programmed cell death (PCD) induced by non-R genes. INF1, encoding a Phytophthora infestans elicitor and Bax, encoding a mammalian protein inducing apoptopic cell death (APC) were co-agro-infiltrated with SIX6. No cell death suppression was observed in either case (Table 1) (data not shown). In summary, Six6, like Avr1, suppresses cell death triggered by the R protein I-2. Interestingly, this effect seems to be I-2 specific as cell death induced by other genes was unaffected by SIX6 co-expression.

**Table 1:** Overview of cell death-inducing proteins tested in the Six6 suppression assay. CNL: Coiled-coil Nucleotide-binding Leucine-rich repeat protein, RLP: Receptor Like Protein.

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<th>Cell death triggering protein</th>
<th>Donor organism</th>
<th>Protein class</th>
<th>Conferring resistance to</th>
<th>Recognition of</th>
<th>Type of cell death (CD)</th>
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**Six6 suppresses ion leakage accompanying I2CD**

Visual scoring of cell death is semi-quantitative. To measure cell death more quantitatively, ion leakage was measured, as it is known to accompany cell death (Coll et al., 2011). SIX6, AVR1 or SIX8 were co-expressed with AVR2 and I-2 using agro-infiltration in *N. benthamiana*. Twenty-six hours after infiltration leaf discs were collected from the
infiltrated area. These discs were transferred to water and conductivity of the solution was recorded for up to 12 h. For the first 10 h the conductivity curves were highly reproducible, but at later time points variation increases (data not shown). Therefore, two representative time points, 2 and 8 h after infiltration, were selected and these are depicted in Figure 6C and D. As negative control an Avr2 variant (Avr2<sup>V→M</sup>) (Houterman et al., 2009) was used that is not recognized by I-2. Co-expression of Avr2<sup>V→M</sup> with I-2 and AVR2 resulted in an identical conductivity curve as co-expression of SIX8, validating its use as a negative control (Fig. S2).

Eight hours after sampling, the AVR1-infiltrated leaf discs showed a significantly (p=0.04) lower conductivity than the Avr2<sup>V→M</sup> control, which is in line with its ability to suppress I2CD (Fig. 6C). Conductivity in the SIX6-infiltrated discs was also significantly reduced at this time point as compared to the Avr2<sup>V→M</sup> control (p=0.002) (Fig. 6D). Hence, cell death suppression by Avr1 and Six6 is accompanied by a significant suppression of I2CD-mediated ion leakage.

**Six6-GFP is present in cell cytoplasm and nucleus**

Determining the subcellular localization of a protein can provide insight in the processes it affects. Hence, the SIX6 gene without the sequence encoding the signal peptide (dSP<sub>SIX6</sub>) was fused to GFP and expressed in <i>N. benthamiana</i> leaves by agro-infiltration. As a reference, GFP alone was expressed using the same vector. The integrity of the Six6-GFP fusion after agro-expression was confirmed by Western blot analysis (Fig 7C). At two dpi, leaves were harvested and fluorescence was visualised using confocal microscopy. In epidermal cells expressing solely GFP, green fluorescence was observed at equal intensities in cytoplasm and nucleus (Fig. 7B). Expression of dSP<sub>SIX6-GFP</sub> also resulted in green fluorescence in both the cytoplasm and the nucleus, however in this case fluorescence appeared stronger in the nucleus than in the cytoplasm, suggesting a nuclear preference for the Six6-GFP fusion protein (Fig. 7A and 2x magnification).

In conclusion, upon agro-infiltration Six6-GFP localizes in both the nucleus and in the cytoplasm suggesting that it is freely diffusible and is not tethered to a membrane or localized in a specific organelle.
Figure 7: Six6-GFP is localized in the cytoplasm and cell nucleus in *N. benthamiana* leaves upon agro-infiltration. Confocal image of leaves agro-infiltrated with (A) dSPSIX6-GFP (left and right at a two times magnification) or (B) GFP alone. Fluorescence in the leaf epidermal cells was determined 24 hpi with confocal microscopy using an excitation wavelength of 488 nm and a 40 times magnification. Each panel consists of a picture taken with a 650 nm long path filter (upper left) for plant autofluorescence, with a 520-550 nm band path filter (upper right) for GFP, transmitted light (lower left) and a stack of all three pictures (lower right). Arrows indicate different cell organelles (Cp cytoplasm, N nucleus, Ch chloroplast). Correct size of the fusion proteins was confirmed by Western blotting of leaf protein extracts using an anti-GFP antibody (C). Ponceau S staining as loading control. M: protein marker.

Discussion

Fol effectors differ in their distribution within the *F. oxysporum* species complex. Whereas some are highly Fol-specific others are also found in other *forme speciales*. Six6 is not only present in other *forme speciales* of *F. oxysporum* but two homologs are also present in *C. higginsianum* and *C. orbiculare*. The Six6 homologs in the *F. oxysporum* complex are more related to Fol Six6 than the ones from *C. higginsianum* and *C. orbiculare*. This might reflect independent evolution of the gene in the genus *Colletotrichum* and *Fusarium* after speciation. However, since Six6 is not found in
other Fusarium or Colletotrichum species nor in other genera, another possibility is that SIX6 was exchanged between Colletotrichum and F. oxysporum after speciation by horizontal gene transfer and then evolved at different rates within the respective fungal species. If so, transfer did not occur recently as their Six6 sequences have considerably diverged. Despite the relatively low conservation of Six6 between the two genera, three conserved C-terminal motifs can be discerned. These motifs might represent regions with conserved structural or biochemical functions and are good targets for future structural and functional analysis.

SIX6 is expressed in planta during infection and is essential for full virulence of Fol. Rather surprisingly, deletion of SIX6 affected merely plant weight but not the disease index of infected tomato plants. Clearly, the SIX6 knockout still has the capacity to cause severe vessel browning when evaluated three-weeks after infection (Fig S1). The high residual plant weight suggests that the infection process is slower, allowing the plant to continue to grow during early stages of infection. This interpretation is supported by the observation that plant weight is highest for plants inoculated with strains ΔSIX6-3 and ΔSIX6-3+SIX6-4, that have a relatively low amount of fungal biomass based on their FEM1 expression (Fig. 4). Remarkably, all Fol effector mutants analysed so far (AVR1, AVR2, AVR3 and SIX6) are compromised in virulence, which implies limited redundancy among Fol effectors. This notion corresponds with the relatively small number of candidate effector genes encoded on the pathogenicity chromosome (16) and the limited number of candidate effector proteins found in the xylem sap (14) (Houterman et al., 2007; Lievens et al., 2009; Ma et al., 2010; Schmidt et al., 2013).

Although Six6 is a virulence factor for Fol, transient expression of SIX6 did not affect growth of the bacterium A. tumefaciens. Also, stable expression of SIX6 in A. thaliana did not alter its susceptibility to the plant pathogenic bacterium P. syringae nor to the fungal pathogens V. dahliae and F. oxysporum. This result could be explained by the presumptive Six6 host target being I) not present in this plant species, II) not present in the cytosol or III) not required for susceptibility to the aforementioned pathogens. Alternatively, IV) these pathogens might carry effectors whose activity is similar to that of Six6, concealing the effect of the expression of this gene. As no Six6 homologs are present in Fo5176, P. syringae or V. dahliae, such effectors would then not be related at the sequence level. We favour the fourth option as we found Six6 to be conserved in F. oxysporum strains infecting diverse hosts such as melon, banana, cotton, cucumber, watermelon, and passion fruit. The gene is also present in C. higginsianum that infects A. thaliana, and C. orbiculare, which causes disease in cucumber and melon, making alternative I) unlikely. An argument to reject II) comes from our suppression assays, in which cytosolic Six6 was found to suppress I2CD in N. benthamiana. Hence, at least the Six6 target involved in I2CD disturbance must be present intracellularly in N. benthamiana. Interestingly, suppression of I2CD seems to be a specific feature of the Six6-target, as no suppression
of cell death was found when SIX6 was co-expressed with other R/AVR gene-pairs, INF1 or the cell death inducing protein Bax (Tab. 1). This finding is surprising as in agro-infiltration assays cell death suppressing effectors typically act more broadly as exemplified by AvrPtoB, HopPtoE and HopPtoF from P. syringae, suppressing cell death accompanying both HR and apoptotic cell death (APC) (Jamir et al., 2004; Abramovitch et al., 2006). Also P. infestans and P. sojae effectors suppress various types of cell death like APC, programmed cell death (PCD) and HR mediated by different R proteins (Dou et al., 2008; Kelley et al., 2010; Gilroy et al., 2011; Wang et al., 2011). Besides effectors from bacteria and oomycetes, also SPRYSEC-19, an effector of the nematode Globodera rostochiensis, suppresses cell death mediated by several CC-NB-LRR immune receptors (Postma et al., 2012). To our knowledge, only the effector CgDN3 from C. orbiculare suppresses specifically only one form of cell death: the one mediated by the secreted protein NIS1 (Yoshino et al., 2012). Possibly, Six6 is a weak suppressor, and I2CD may be relatively easy to suppress as I-2 mediated resistance to AVR2-carrying Fol strain typically does not involve HR (Beckmann, 2000). The I2CD observed in N. benthamiana might be an anomaly due to overexpression of I2-AVR2 in this heterologous system, resulting in a cell death response, which may be easier to suppress than that induced by R genes that do rely on HR for their function (Thomma et al., 2011).

With the host target of Six6 remaining unidentified, we can only speculate on how Six6 contributes to virulence of Fol on susceptible plants. The localization of GFP-tagged Six6 in nucleus and cytoplasm N. benthamiana leaves upon transient expression suggests that the effector target could be localised in either, or both, of these subcellular compartments. It has been shown that I-2 activation requires Avr2 recognition in the nucleus (Ma et al., 2013) and nuclear localised Six6 might interfere with this event, thereby suppressing I2CD. Yet, its capacity to suppress I2CD is insufficient to suppress I-2 function in tomato, as I-2 plants are resistant to SIX6-expressing Fol strains. However, I-2-mediated resistance to Fol is not absolute and growth of the fungus in the xylem vessels of roots and stems of resistant plants is frequently observed (Elgersma et al., 1972; our unpublished results). Possibly, some of this fungal growth is due to partial, Six6-mediated suppression of I-2, implying that fungal growth of a SIX6 knockout on a resistant plant will be reduced. Unfortunately, the reduction of fungal biomass already observed upon infection of susceptible plants by the SIX6 knockouts (Fig. 4) prevented us from testing this hypothesis. This virulence phenotype on susceptible plants also suggests that Six6 has other effects on host susceptibility than (partial) suppression of I-2-mediated host responses.

Future work should include experiments aimed at identification of the Six6 host target(s), to uncover how this effector contributes to virulence of Fol towards tomato and suppression of I2CD in N. benthamiana. It will also be interesting to see whether the Six6 homologs found in other plant pathogens are also required for full pathogenicity.
Materials and Methods

Alignment

The Six6 protein alignment was performed with ClustalW and Seaview (http://www.ebi.ac.uk/Tools/msa/clustalw2/ and http://pbil.univ-lyon1.fr/software/seaview.html).

Plant material, fungal and bacterial strains

Tomato variety C32 was used for the Fol-inoculation assays and is susceptible to all Fol races (Kroon and Elersoma, 1993). A. thaliana ecotype Col-0 was used for transformation and the disease assays with V. dahliae JR2 (kindly provided by B. Thomma, WUR, Wageningen, NL), P. syringae pv. tomato DC3000 (Whalen et al., 1991) and Fo5176 (originally isolated by Queensland Plant Pathology Herbarium, Queensland Department of Primary Industries and Fisheries, Brisbane, Australia). All Fol-gene deletion lines were generated in a Fol007 background (kindly provided by L. Davidse (France)), a race 2 isolate non-pathogenic on tomato plants carrying I-2 or I-3.

Agro-infiltrations were performed using the disarmed A. tumefaciens strain GV3101::pMP90 (Koncz and Schell, 1986). A. tumefaciens mediated transformation of Fol was done with the strain EHA105 (Hood et al., 1993) and for A. thaliana transformation the strain Agl-0 was used (Lazo et al., 1991).

RNA isolation and RT-PCR

Ten-day-old tomato seedlings were inoculated with Fol007, the SIX6 knockout strain or the knockout strain complemented with SIX6 with the root dip method (Wellmann, 1939) and then potted in vermiculite (Agra-vermiculite, Eveleens, Aalsmeer, the Netherlands). Ten days after inoculation the roots were cut below the hypocotyls, rinsed twice with water and dried with tissues. The root samples were ground in liquid nitrogen. Total RNA from the samples was extracted with TRIzol LS reagent (Invitrogen) and subsequently purified with RNeasy Mini kit (Qiagen). DNA was removed by on-column treatment with RNase-free DNase (Qiagen). cDNA was synthesized using the M-MuLV reverse transcriptaseRNase H minus kit (Fermentas, Thermo Scientific). Either primer pair FP1995/FP1996 or FP2155/FP2156 (list of all primer sequences Tab. S3) were used to amplify SIX6 from cDNA. FEM1 was amplified using primer pair FP157/FP158.

To assess SIX6 expression of Fol grown in artificial medium 0,5 ml of 10^7 spores/ml of Fol4287 (race 2) were added to 4,5 ml of a one-week-old tomato MSK8 cell culture grown in BY-medium (Felix et al., 1991) or to NO_3-medium (100 mM KNO_3, 3% sucrose and 0.17% Yeast Nitrogen Base without amino acids or ammonia). After 24 hours of
incubation at 22°C, the material was harvested, washed two times with sterile water and freeze-dried. RT-PCR was performed as described above but DNase (Fermentas) treatment was executed before cDNA synthesis.

**Construction of the SIX6 knockout- and complementation-constructs**

To generate a SIX6 gene deletion, a 1328 bp upstream sequence (starting 36 bp 5' of the SIX6 start codon) was amplified by PCR using primers FP1509 and FP1510, carrying either an XbaI or a HindIII site. The PCR product was inserted into the binary vector pRW2h (Houterman et al., 2008) upstream of the hygromycin resistance cassette. An 836 bp fragment starting 124 bp after the SIX6 start codon containing an internal BssHII site was amplified using the primers FP1511 and FP1512 and BssHII digested. The resulting 676 bp fragment was inserted into a StuI/BssHII digested vector downstream of the Hygromycin cassette.

The complementation construct was generated using primers FP1727 carrying a BcuI (SpeI) linker and FP1728. PCR amplification resulted in a 1.9 kb fragment starting 1023 bp upstream of the SIX6 start codon and ending 261 bp downstream of the stop codon. The fragment was integrated into a XbaI/Eco147I (StuI) digested pRW1p vector.

**Construction of binary vectors for agro-infiltration**

SIX6 was PCR-amplified from genomic DNA of Fol007 (race 2). Forward primer FP2423 generated a truncated SIX6 gene lacking the sequence encoding its signal peptide. To allow C-terminal tagging of Six6, a PCR-amplification was also done with the FP2204 reverse primer that removes the stop-codon. To generate a similar fragment for SIX8, the gene was amplified using primers FP2838 and FP2206 from cDNA isolated from Fol007-infected tomato roots. AVR1 lacking the sequence encoding it’s signal peptide and prodomain was obtained by PCR-amplification using primers FP2292 and FP2202 on cDNA of Fol001 (race 1)-infected tomato roots. To all PCR products Gateway attB flanks were added in a subsequent PCR-amplification with primers FP872 and FP873. The obtained attB PCR products were recombined via entry clone pDONR207 (invitrogen) into binary vectors CTAPi (Rohila et al., 2004) and pGWB451 following the Gateway protocol (Invitrogen). All primers were purchased from MWG (Germany). AVR2 and I-2 carrying constructs have been described previously (van Ooijen et al., 2008; Houterman et al., 2009).

**Agro-infiltration of N. benthamiana**

Agro-infiltrations were performed according (Ma et al., 2012) as described. In brief, A. tumefaciens strain GV3101 carrying the construct of interest was grown until OD\textsubscript{600} 0.8
in LB$_{\text{tum}}$ medium supplemented with 20 µM acetylsyringone and 10 mM MES, pH 5.6. The cells were resuspended in infiltration medium (5g/l MS salts, 20g/l sucrose, 200 µM Acetylsyringone, 10 mM MES pH 5.6) to a final OD$_{600}$ 1.0 when used for in planta protein expression or localization studies. For suppression assays A. tumefaciens suspensions were diluted to an OD$_{600}$ of 0.5 or 0.3.

**Protein Extraction and Western blotting**

To determine accumulation of GFP- or TAPi-tagged recombinant proteins, agro-infiltrated N. benthamiana leaves were harvested 24 – 48 hours post infiltration (hpi) and frozen in liquid nitrogen. Protein extraction was done as described previously (van Ooijen et al., 2008; Ma et al., 2012), 0.5 g of frozen plant tissue was ground in liquid nitrogen and allowed to thaw slowly in 1ml extraction buffer (25 mM Tris pH 7.5; 1 mM EDTA, 150 mM NaCl; 5 mM DTT; 1x Roche Complete protease inhibitor cocktail; 2 % PVPP; 0.1 % NP-40). The extract was centrifuged at 13000 rpm at 4°C for 30 min and the supernatant was filtered through miracloth (http://calbiochem.com). The protein concentration was determined by measuring the OD$_{280}$ on a spectrophotometer (Hitachi U-2900) and equal amounts of proteins were loaded on a 12% SDS-polyacrylamide gel. For protein detection proteins were wet blotted on PVDF membranes and equal loading and transfer was confirmed by staining the blot with 2 % Ponceau S in 5 % acetic acid. Subsequently, membranes were blocked with 5 % milk in Tris-buffered saline (TBS) and GFP or TAPi-tag fusion proteins were detected with a @GFP- (VXA6455, Invitrogen) (1:3000 diluted) or @PAP-antibody (Sigma P1291) (1:5000 diluted), which is linked to horseradish peroxidase. In case of GFP-detection a goat-@rabbit antibody (Pierce P31430) (1:8000 diluted) was used as secondary antibody. In either case the signal was detected using enhanced chemiluminescence (ECL) (ECL Plus Western blotting Detection System (GE Healthcare)).

**Confocal microscopy**

In planta accumulation of Six6-GFP after agro-infiltration was visualised using confocal microscopy (Zeiss LSM 510). An Ar-laser (intensity set at 22 %) was used for excitation at 488 nm. Fluorescence was detected with a 520-550 nm band path filter for GFP and autofluorescence of the plant tissue was recorded using a 650 nm long path filter. Scans were taken with the C-Apochromat 40X1.2 Wcorr objective.

**Visualization and quantification of the hypersensitive response**

For quantification of cell death three leaf discs with a diameter of 7 mm were collected per agro-infiltrated sector 27 h after agro-infiltration. The leaf discs were washed in 50 ml water for 30 min while shaking at 100 rpm. Afterwards the discs were placed,
with their abaxial sides facing downwards, in a well containing 3 ml 0.01% Silwet-L77. The leaf discs were vacuum-infiltrated and the 12 well-culture plates (Greiner) were incubated at room temperature under TL-light (25 lux) while shaking at 100 rpm. 25 µl of the solution was sampled every 1 to 2 h for 12 h and conductivity was determined with a TwinCond conductivity meter (Horiba).

To visualize cell death leaves were stained with Trypan Blue (Ma et al., 2012). Thereto 50 ml of staining solution (100 ml lactic acid (DL Sigma L-1250), 100g phenol, 100 ml glycerol, 100 ml H₂O, 100 mg trypan blue) was mixed with 50 ml 96% EtOH and heated in a water bath until boiling. Up to 3 leaves were added together to the boiling solution and allowed to stain for 5 min. Leaves were destained with 2.5 mg/ml chloral hydrate solution overnight and scanned using an Perfection 1200 Scanner (Epson, USA).

**A. tumefaciens growth curves**

*A. tumefaciens* growth curves were measured as described (Hann and Rathjen, 2007). *A. tumefaciens* GV3101 carrying the appropriate constructs was agro-infiltrated at an OD$_{600}$ of 0.05. Two leaf discs were harvested from each agro-infiltrated area 0, 2 or 4 days post-inoculation. Bacteria were extracted in 500 µl 10 mM MgSO$_4$ after homogenising the leaf material by adding two metal beads (2.5 mm diameter) and placing the tube for 2.5 min in a paint shaker (Fast and Fluid management, IDEX Corporation). Serial dilutions of the homogenate were made and 20 µl of each dilution (typically $10^{-3}$, $10^{-4}$, $10^{-5}$) was plated on LB$_{tum}$ (10 g l$^{-1}$ tryptone, 5 g l$^{-1}$ yeast extract, 2.5 g l$^{-1}$ NaCl) supplemented with Rifampicin (20 µg/ml). After two days of incubation at room temperature the colony forming units (cfu) were counted and the cfu/cm$^2$ valued was calculated. Each sampling was performed in three technical and five biological replicates. Significance was tested with a paired student t-test (p < 0.05).

**Plant inoculations**

Ten-day-old tomato seedlings were uprooted from the soil. The seedlings were placed for 5 min in a five-day-old Fol spore suspension (10$^7$ spores/ml) and potted. Disease progression was evaluated after three weeks. Thereto plant weight and disease index (Rep et al., 2004) were scored (Fig. S1) of 20 plants/treatment. Significant differences between treatments were tested using ANOVA.

Inoculation of *A. thaliana* plants with *V. dahliae* JR2 and scoring of the disease index was done as described (Yadeta et al., 2011). *A. thaliana* was grown under short day conditions in a climate chamber (21°C/70% relative humidity/11 h photoperiod). After 21 days the plants were carefully removed from the soil and the roots were placed in a *V. dahliae* spore suspension (10$^6$ spores/ml) for 5 min. Thereafter the plants were transferred to fresh soil. After two to three weeks the first disease symptoms
developed. Disease symptoms were scored visually by measuring the size of the plants and evaluating development of chlorosis on the leaves (Yadeta et al., 2011).

5 ml of the spore suspension (10⁶ spores/ml) of a *F. oxysporum* Fo5176 culture was added directly to the soil of 13-day-old *A. thaliana* seedlings growing on a soil/sand mix (ratio 1:1). The disease index was scored on a scale from 0 (no disease symptoms), 1 (one or two leaves with yellow vessels), 2 (lowest full developed leaves show chlorosis), 3 (all full developed leaves show chlorosis), 4 (all leaves show chlorosis, including the rosette of new developed leaves) to 5 (plant is dead) at nine and 15 days after inoculation (Tab. S2).

*Pseudomonas syringae* was cultured overnight in King’s B medium (King et al., 1954) and resuspended in 10 mM MgSO₄ to a final OD of 0.0007. The bacteria were syringe-infiltrated into the leaves of 22-day-old *A. thaliana* plants. Three days after inoculation leaf discs (0.5 cm) were collected and the bacteria were extracted in 10 mM MgSO₄. Different dilutions (typically 10⁻³, 10⁻⁴, 10⁻⁵) of the bacteria suspension were plated on King’s B.

**Transformation of Fol, targeted deletion and complementation of SIX6**

Transformation of Fol was mediated by *A. tumefaciens* transformation (Takken et al., 2004). Thereto spores of a five-day-old Fol culture were isolated and resuspended in IM (10 mM glucose, 10 mM K₂HPO₄, 10 mM KH₂PO₄, 2.5 mM NaCl, 4 mM (NH₄)₂SO₄, 0.7 mM CaCl, 2 mM MgSO₄, 9 µM FeSO₄, 0.5 % (w/v) glycerol, 40 mM MES pH 5.3) supplemented with 200 µM acetosyringone to a final concentration of 2·10⁶ spores/ml. *A. tumefaciens* containing the binary vector of interest was grown for 24 h in 2x Yeast extract and Tryptone medium (2x YT) supplemented with the appropriate antibiotics. Then the cells were resuspended in 10 ml IM to OD 0.45 and incubated for 6 h at 28°C. 2 ml of each of the Fol and the *A. tumefaciens* resuspensions were mixed and aliquots of 25 µl were transferred to ME25 filters placed on co-cultivation plates (same as IM but containing 5 mM glucose) solidified with 1.5 % Bacto-agar. After two days of incubation at 25°C the filters were transferred to CDA (Czapek Dox Agar) containing cefotaxime and hygromycin or zeocin (both 100 µg/ml). Plates containing with zeocin were buffered with 0.1 M Tris, pH 8.0. Putative transformants appeared after two to four days and monospores were made for further analysis. Absence of SIX6 in hygromycin resistant transformants was tested using primer pair FP1490/FP1491. In locus insertion of the knockout cassette was confirmed by PCR using primer pairs FP745/FP2264 (right border) and FP2266/FP659 (left border). Presence of SIX6 in the SIX6 knock-out complementation strains was also confirmed by PCR using three primer pairs, of which one (FP745/FP1961) amplified the region upstream of SIX6 and a part of the SIX6 coding region, and the other two primer sets (FP1995/FP2218 and FP1959/FP746) amplified the region downstream of SIX6 and a part of the SIX6 coding region.
Transformation of *A. thaliana* using the floral dip method

*A. thaliana* plants were grown in a 12 h photoperiod at 70% relative humidity and 21°C. The first inflorescence was removed to boost flower production. *A. tumefaciens* carrying the dSP*SIX6-TAPi* was grown in 250 ml LB$_{\text{tum}}$ supplemented with spectinomycin (100 µg/ml) over night. The bacteria were resuspended in 5 % sucrose to a final OD 0.8 and 0.02 % Silwet was added. The flowers were dipped in the bacterial suspension, wrapped in plastic foil to increase humidity and protected against direct light. The dipping was repeated after one week. After seed harvest and sterilization the seeds were plated on MA plates (2.2g/l MS incl. gamborg B5 vitamines, 36.7 mg/l FeNaEDTA, MES, pH 5.8, 0.7 % Daishin agar) containing 100 µg/ml nystatin and 10 µg/ml phosphinothricin. Resistant plants were propagated and homozygous plant lines carrying single *SIX6* T-DNA insertions were selected after segregation analysis. Six6 protein expression was analysed by extracting the proteins from leaf material (100 mg) in urea buffer (9.5 M Urea, 100 mM Tris pH 6.8, 2 % SDS, 2% β-mercaptoethanol) following detection of their attached TAPi-tag by western blot analysis.

**Acknowledgments**

We thank T. Drost for assisting with the *Pseudomonas* and *Agrobacterium* bioassays, T. Hendrix, H. Lemereis and L. Tikovsky for greenhouse management and A. Pietraszewska and R. Breedijk for assistance with fluorescence microscopy.

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Supplementary data

Table S1 related to Figure 3: List showing clusters (a-f) of treated plants based on the plant weight or disease index. Significance was tested with ANOVA (Fisher PLSD significant at 95%).

<table>
<thead>
<tr>
<th>Weight</th>
<th>Disease index</th>
</tr>
</thead>
<tbody>
<tr>
<td>mock</td>
<td>a</td>
</tr>
<tr>
<td>ΔSIX6-1</td>
<td>b</td>
</tr>
<tr>
<td>ΔSIX6-2</td>
<td>c</td>
</tr>
<tr>
<td>ΔSIX6-3</td>
<td>d</td>
</tr>
<tr>
<td>ΔSIX6-4</td>
<td>e</td>
</tr>
<tr>
<td>ΔSIX6-5</td>
<td>f</td>
</tr>
<tr>
<td>Fol007</td>
<td>g</td>
</tr>
<tr>
<td>ect-1</td>
<td>h</td>
</tr>
<tr>
<td>ect-2</td>
<td>i</td>
</tr>
</tbody>
</table>

Table S2: related to figure 5D: Disease index of *Arabidopsis thaliana* plants infected with Fo5176.

<table>
<thead>
<tr>
<th>Disease index</th>
<th>Symptoms 9 dpi</th>
<th>Symptoms 15 dpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>no symptoms</td>
<td>no symptoms</td>
</tr>
<tr>
<td>1</td>
<td>plant smaller, mild chlorosis of a few vessels</td>
<td>normal size, a few yellow vessels</td>
</tr>
<tr>
<td>2</td>
<td>clear chlorosis of the vessels</td>
<td>reduced size, clear yellow vessels</td>
</tr>
<tr>
<td>3</td>
<td>chlorosis of the vessels, completely yellow leaves</td>
<td>reduced size, yellow vessels, leaves with full chlorosis</td>
</tr>
<tr>
<td>4</td>
<td>whole plant chlorotic, sometimes fungus growing out</td>
<td>very small plants, only top leaves still green, no fungus</td>
</tr>
<tr>
<td>5</td>
<td>plant is dead</td>
<td>fungus all over the plant, or plant dead</td>
</tr>
</tbody>
</table>
**Table S3**: List of oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Oligomer sequence</th>
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</thead>
<tbody>
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<td>ATGAAGTACACTTCGCACGCTACC</td>
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<td>FP158</td>
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<tr>
<td>FP659</td>
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<tr>
<td>FP745</td>
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<tr>
<td>FP746</td>
<td>TGGGCAACTTGGGGAAGAGG</td>
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<tr>
<td>FP872</td>
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<td>FP873</td>
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<td>FP1491</td>
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<td>FP2838</td>
<td>AAAAAAGGAGGCTTCCATGAGGCTATGATAAGG</td>
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**Figure S1**: Fol disease index (DI) scores. Typical examples of (A) Fol-infected tomato plants representing the disease index classes 0 to 4 (DI-D4) and (B) cross section of the stems cut just underneath the cotyledons (upper row) or at the height of the cotyledons (lower row). 0 = no symptoms, 1 = one or two brown vascular bundles in the stem below the level of the cotyledons, 2 = one or two brown vascular bundles at the level of the cotyledons (no strong growth distortion, but plants can be smaller), 3 = at least three brown vascular bundles and growth distortion (strong bending of the stem and asymmetric development), 4 = all vascular bundles are brown, plant either dead or small and wilted.
Six8 and $AVR2^{V\rightarrow M}$ do not suppress ion leakage. Conductivity assay performed with agro-infiltrated *N. benthamiana* leaf discs. $AVR2$, I-2 and SIX8 or AVR2, I-2 and $AVR2^{V\rightarrow M}$ were co-expressed on different halves of the same leaves and leaf discs were sampled at 26 hpi. Bars represent standard deviation (N=5). No significant differences in ion leakage were observed between Six8 and $Avr2^{V\rightarrow M}$ in a student t-test (p<0.05)
References


Characterization of Six6


